Applied and Environmental Microbiology, Sept. 1998, p. 3352–3358 Vol. 64, No. 9
0099-2240/98/$04.00+0

Direct Determination of Carbon and Nitrogen Contents of Natural Bacterial Assemblages in Marine Environments

RUMI FUKUDA,* HIROSHI OGAWA, TOSHI NAGATA, and ISAO KOIKE

Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164-8639, Japan

Received 30 January 1998/Accepted 17 June 1998

In order to better estimate bacterial biomass in marine environments, we developed a novel technique for direct measurement of carbon and nitrogen contents of natural bacterial assemblages. Bacterial cells were separated from phytoplankton and detritus with glass fiber and membrane filters (pore size, 0.8 μm) and then concentrated by tangential flow filtration. The concentrate was used for the determination of amounts of organic carbon and nitrogen by a high-temperature catalytic oxidation method, and after it was stained with 4′,6-diamidino-2-phenylindole, cell abundance was determined by epifluorescence microscopy. We found that the average contents of carbon and nitrogen for oceanic bacterial assemblages were 12.4 ± 6.3 and 2.1 ± 1.1 fg cell^{-1} (mean ± standard deviation; $n = 6$), respectively. Corresponding values for coastal bacterial assemblages were 30.2 ± 12.3 fg of C cell^{-1} and 5.8 ± 1.5 fg of N cell^{-1} ($n = 5$), significantly higher than those for oceanic bacteria (two-tailed Student's t test; $P < 0.03$). There was no significant difference ($P > 0.2$) in the bacterial C:N ratio (atom atom^{-1}) between oceanic (6.8 ± 1.2) and coastal (5.9 ± 1.1) assemblages. Our estimates support the previous proposition that bacteria contribute substantially to total biomass in marine environments, but they also suggest that the use of a single conversion factor for diverse marine environments can lead to large errors in assessing the role of bacteria in food webs and biogeochemical cycles. The use of a factor, 20 fg of C cell^{-1}, which has been widely adopted in recent studies may result in the overestimation (by as much as 330%) of bacterial biomass in open oceans and in the underestimation (by as much as 40%) of bacterial biomass in coastal environments.

Because bacterioplankton play important roles in the cycling of carbon and nitrogen within marine environments (1, 14, 16), it is essential to assess bacterial biomass accurately in order to better understand food webs and biogeochemical fluxes. Previous studies have suggested that the carbon biomass of bacteria generally exceeds that of phytoplankton in oligotrophic oceans (11, 16, 20). These studies have estimated bacterial biomass based on the assumption that one marine bacterial cell contains 20 fg of carbon. This value was determined with coastal bacterial assemblages grown in filtered seawater (29) and has been commonly applied to oceanic bacterial assemblages without much critical confirmation (11, 16, 25, 31). Recently, Christian and Karl (12) examined the biomass distribution of microbial communities in the subtropical Pacific Ocean by using biomass indicators of microorganisms and a least-squares inverse method. They suggest that the average carbon content of bacteria in the investigated area could be close to 10 fg per cell, a value which is much lower than commonly used factors. However, carbon contents of natural populations of oceanic bacterioplankton have yet to be determined directly.

Previous studies have determined conversion factors by dividing bacterial carbon by bacterial biovolume (3, 5, 18). Bacterial carbon is measured as particulate organic carbon retained on a glass fiber filter, and biovolume is estimated by measuring bacterial size by epifluorescence microscopy (7, 29, 33, 41), electron microscopy (7, 30, 43), or particle counting (26). Bacterial assemblages have commonly been preincubated to minimize the effects of detritus and phytoplankton (34). The reported carbon-to-biovolume factors of marine bacteria vary widely, ranging from 0.18 to 1.61 pg of C μm^{-3} (6, 26, 28, 29); some of these values are unrealistically high (4). The variability may be related to errors in size measurement (28–30, 34), differences in the taxonomic compositions and physiological states of bacterial assemblages (28), or both. This large variability should introduce significant errors into estimates of bacterial biomass calculated from cell number, average cell size, and carbon-to-biovolume ratio. In addition, preincubation before measurement could change the compositions of bacterial populations (15, 22).

In this paper, we report our technique for estimating the carbon and nitrogen contents of natural bacterial assemblages in oceanic and coastal environments. Natural bacterial populations, separated from phytoplankton and detritus, were concentrated by tangential flow filtration. Carbon and nitrogen contents of bacterial cells were determined by the high-temperature catalytic oxidation (HTCO) method. Our novel approach made possible, for the first time, the direct determination of the carbon and nitrogen contents of natural bacterial assemblages in oligotrophic marine environments.

MATERIALS AND METHODS

Sampling. Oceanic samples were collected in the Southern and Pacific Oceans during a cruise (leg III to IV of KH-94-4, 10 January to 14 February 1995) of the R. V. *Hakuho-maru* (see Table 1). In the Southern Ocean (south), the water sample was collected at a depth of 40 m with a Niskin sampler (volume, 12 liters). In the other oceans a diaflame pump was used to collect water samples, while the ship was steaming. The inlet of the pump was at about 4 m below the surface. Surface coastal samples were collected from the Tokyo Bay and the Otsuchi Bay with a Van Dorn water sampler (see Table 1). The vertical variability of bacterial carbon and nitrogen contents was not examined in this study.

Prefiltration and concentration procedures. We tested several combinations of prefiltration filters for removal of phytoplankton and other large particles from the sample waters. In the prefiltration process, ca. 100 to 600 liters of samples were filtered through 30-cm^2 glass fiber filters (GA-200 for the Tokyo Bay and GF-75 for all other stations; TOYO ROSHI Corp., Tokyo, Japan) by gravity. Although the nominal pore sizes provided by the manufacturer for the GA-200 and GF-75 filters are 0.8 and 0.3 μm, respectively, a significant portion of coccoid cyanobacteria passed through these glass fiber filters, except for the

* Corresponding author. Mailing address: Ocean Research Institute, University of Tokyo, 1-15-1 Minami-dai, Nakano, Tokyo 164-8639, Japan. Phone: 81-3-5351-6457. Fax: 81-3-5351-6461. E-mail: rfukuda@ori.u-tokyo.ac.jp.

Otsuchi Bay samples. Therefore, the oceanic samples and Tokyo Bay samples were further filtered through a cartridge membrane filter (Micropore 8AU, type BS; nominal pore size, 0.8 μm; size, 7 by 25 cm; Organo Corp., Tokyo, Japan).

Particles in the prefiltered seawater were concentrated to approximately 100 ml in volume with a Pellicon tangential flow filtration system (Millipore Corp., Bedford, Mass.) with 0.465 m^2 of a 0.1-μm-pore-size Durapore membrane (hydrophilic polyvinylidene difluoride; Millipore Corp.) attached (see Table 2). For the Tokyo Bay sample, we used a hollow-fiber cartridge filtration system to which 0.03 m^2 of a 0.1-μm-pore-size PM membrane (polysulfone; Amicon, Inc., Beverly, Mass.) was attached. These systems were run by a peristaltic pump with filter velocities of ca. 500 ml min^{-1} and a pressure of less than 1 kg cm^{-2}. After a concentrate was recovered, a small volume of sodium chloride solution (ca. 200 ml) was flushed through the system to wash out bacteria adsorbed to the membrane. This solution was prepared with precombusted sodium chloride and Milli-Q water, and the salinity was adjusted to that of the samples. We repeated this washout procedure four to five times with a total volume of approximately 1 liter and combined all of the concentrated suspensions. Oceanic-sample suspensions were further concentrated to less than 100 ml by using a cell stir filtration system (model 202; Amicon, Inc.) with a 0.1-μm-pore-size Durapore membrane. The system was washed with approximately 200 ml of sodium chloride solution to recover the bacterial concentrate. The Otsuchi Bay samples obtained with a Pellicon system were further concentrated by centrifugation (at 15,000 × *g* or 21,000 × *g* for 1 h) after samples were weakly sonicated for 1 to 3 min in a sonication bath (IC 92; Branson Ultrasonics Corp., Danbury, Conn.) to disperse bacterial aggregates and detrital particles (44).

Enumeration of phytoplankton and bacterial abundance. Chlorophyll *a* was extracted with *N*,*N*-dimethylformamide after suspended materials in the samples were collected on Whatman GF/F filters (25 mm) and measured with a fluorometer (model 10R or 10-AU-005; Turner Designs, Sunnyvale, Calif.) (40). Ten-milliliter volumes of bacterial subsamples were preserved with buffered formalin (2%, vol/vol) and stored at 4°C in the dark until the preparation of microscope slides (within 1 week). Cells were stained with 4′,6-diamidino-2-phenylindole, filtered on Irgalan black-stained 0.2-μm-pore-size Nuclepore filters (Costar, Cambridge, Mass.), and counted by epifluorescence microscopy (Axioplan; Zeiss) (36). At least 240 cells were counted for each filter. The precisions for estimates of bacterial abundance were generally within 15% (coefficient of variation [CV]). Cyanobacteria were counted by epifluorescence microscopy using blue excitation. Counts were repeated 25 times for each filter, and total counts of 0 to 1,860 cells per filter were achieved.

Measurement of organic carbon and nitrogen. Amounts of organic carbon and nitrogen in the concentrated liquid samples were determined by the HTCO method. This method has advantages over the more conventional method of measuring bacterial carbon and nitrogen with an elemental (CHN) analyzer after collecting particles on glass fiber filters (29). First, we can avoid the loss of bacteria that pass through glass fiber filters. Second, the HTCO method is more sensitive and requires less sample by volume (100 μl for an analysis) than CHN analysis (usually >10 ml). HTCO analysis for simultaneous measurements of organic carbon and nitrogen was conducted by using a modified total-organic-carbon analyzer (model TOC-5000; Shimadzu Corp., Kyoto, Japan) equipped with an aluminum oxide catalyst with 3% platinum, to which chromium oxide was added (35). Inorganic carbon was removed from the acidified samples by sonication in a sonication bath (for 10 min; model 14; Branson Ultrasonics Corp.) under a flow of nitrogen gas on the surfaces of the samples (400 ml min^{-1}). This method, instead of more conventional bubbling (42), was used to prevent the formation of organic flocs. Nitrogen gas was prewashed with a barium hydroxide solution to eliminate carbon dioxide contamination. Because a portion of inorganic carbon sometimes remained in the samples after the above procedure (usually less than 10% of the initial amount), the concentration of organic carbon was obtained as the difference between the total- and inorganic-carbon measurements. Inorganic carbon was detected on a TOC-5000 analyzer without combustion, while total carbon was detected after combustion. The procedural blank of organic carbon and total nitrogen was obtained by using a sodium chloride solution which had been used for suspension of bacterial concentrates, except for several oceanic samples, for which Milli-Q water was substituted. The samples were kept frozen at −20°C in sealed 10-ml glass ampoules until analysis. The precision (standard deviation [SD]) of total-carbon analysis was 0.4 to 8.6 μM (CV, 0.1 to 4.6%), and that of total-nitrogen analysis was 0.2 to 1.9 μM (CV, 0.5 to 9.8%).

To examine oxidation efficiency for measuring carbon and nitrogen contents of bacterial samples by our system, a comparison was made between the TOC-5000 analyzer and an elemental analyzer (CHN analysis; model NA 1500 NCS; Fisons Instruments, Milan, Italy). Two bacterial strains (*Marinomonas communis* and *Listonella* [*Vibrio*] *anguillarum*) and the samples from the Tokyo Bay and the Otsuchi Bay were used. Bacterial strains were cultured in half-strength Zobell 2216E medium and harvested during stationary phase. Cultured bacteria do not represent the characteristics of natural assemblages, but they were used in order to obtain a sufficient amount of carbon and nitrogen for CHN analysis. After the cells were washed and suspended in sodium chloride solution, subsamples were kept in ampoules for HTCO analysis (HTCO total). Other portions of samples (50 ml) were filtered through precombusted glass fiber filters (Whatman GF/F) for CHN analysis. The GF/F filtrate was kept in ampoules and used for HTCO analysis (HTCO filt). Carbon and nitrogen values determined by the HTCO method (HTCO total minus HTCO filt) were compared with values obtained by CHN analysis.

RESULTS

Comparison between HTCO and CHN analyses. We compared the amounts of bacterial carbon and nitrogen measured by the HTCO method to those measured by CHN analysis. The relationships between the values obtained by those two methods were described as $y = (0.92 \pm 0.08) \times x - (33 \pm 20)$ for carbon ($r^2 = 0.92$) and $y = (0.94 \pm 0.04) \times x - (4.1 \pm 1.8)$ for nitrogen ($r^2 = 0.98$), where *x* and *y* are the amounts of carbon or nitrogen (in micromolar concentrations) measured by CHN and HTCO, respectively (± standard error; $n = 15$). For both carbon and nitrogen, the slope was not significantly different from 1 ($P > 0.05$ by the two-tailed Student *t* test). The difference between the *y* intercept and zero was not significant (for carbon, $P > 0.1$) or only marginally significant (for nitrogen, $P = 0.04$). Therefore, we concluded that bacterial carbon and nitrogen contents determined by the HTCO method did not differ from those determined by CHN analysis.

Removal of phytoplankton by prefiltration processes. The abundances of bacteria and phytoplankton before and after prefiltration are summarized in Table 1. The removal of phytoplankton was assessed by determining the chlorophyll *a* concentration and the abundance of cyanobacteria. The chlorophyll *a* concentration before prefiltration was 0.078 to 0.49 μg $liter^{-1}$ in oceanic samples and 3.6 to 11.8 μg $liter^{-1}$ in coastal samples. The bacterial abundance of coastal samples (2.0×10^6 to 6.1×10^6 cells ml^{-1}) was 1 order of magnitude higher than that of oceanic samples (2.9×10^5 to 8.3×10^5 cells ml^{-1}). On the other hand, the abundance of cyanobacteria (most of which were coccoid) in oceanic waters was very low in the Southern Ocean (south) ($<0.03 \times 10^3$ cells ml^{-1}) and relatively high in the equatorial Pacific Ocean (3.5×10^4 cells ml^{-1}), where it was almost as high as that in the Otsuchi Bay in June 1995 (2.3×10^4 to 3.7×10^4 cells ml^{-1}) (Table 1).

After filtration with membrane filters (Micropore 8AU), chlorophyll *a* concentrations of oceanic samples were close to the detection limit (0.001 μg $liter^{-1}$) and were less than 1% of concentrations before filtration, except for two subtropical samples from the Pacific Ocean (Table 1). Oceanic bacteria mostly passed through Micropore 8AU filters (87 to 96%), whereas most cyanobacterial cells (>90%) were retained on these filters (Table 1). With coastal samples, chlorophyll *a* concentrations after prefiltration were less than 5% of concentrations before prefiltration. The abundance of recovered bacteria in filtrates was 70 to 80% of that before filtration in the Tokyo Bay and 1995 Otsuchi Bay samples, whereas the abundance was about 30% of that before filtration in the 1996 Otsuchi Bay samples.

Concentrating bacteria by tangential flow filtration. By using approximately 100 to 600 liters of sample, tangential flow filtration achieved a 7- to 200-fold concentration of natural bacterial assemblages (Table 2). The total number of bacteria recovered in the concentrate (retentate) was 4.5 to 84% of the initial number. The numbers of bacteria recovered in the filtrate (permeate) were 7.3 to 68% and 0.02 to 31% of the initial numbers for oceanic and coastal samples, respectively. The total recovery (retentate plus permeate) of bacteria after tangential flow filtration was 13 to 96% (average, 60%) for oceanic samples and 9.3 to 83% (average, 41%) for coastal samples. The concentrated samples, with the exception of that from the Tokyo Bay, were further concentrated by cell stir filtration (for the oceanic samples) or centrifugation (for the Otsuchi Bay samples) before measurement of organic carbon

TABLE 1. Separation of phytoplankton and bacteria by prefiltration[a]

Sample	Position[b]	Sampling depth (m)	Date	Filter type[c]	Chl. *a*		Cyano		Bacteria		Carbon ratio (%)[e] based on:	
					Concn in SW (μg liter^{-1})	% in filtrate[d]	No. in SW (10^3 ml^{-1})	% in filtrate[d]	No. in SW (10^5 ml^{-1})	% in filtrate[d]	Chl. *a* data	Cyano data
Oceanic												
Southern (south)	65°24′S, 140°40′E	40	18 June 1995	8AU	0.48	0.37	<0.03	—[f]	2.9	93	1.4	<0.01
Southern (north)	48°32′S, 146°23′E and 47°46′S, 146°41′E	4	25 June 1995	8AU	0.49	0.65	8.0	<0.4	6.0	96	4.3	<0.2
Subtropical Pacific (south)	14°52′S, 155°10′E and 12°59′S, 154°53′E	4	4 Feb. 1995	8AU	0.078	17.8	0.97	<6	5.8	91	11	<2
Subtropical Pacific (north)	15°20′N, 141°24′E and 16°27′N, 140°20′E	4	10 Feb. 1995	8AU	0.10	8.2	0.67	<10	6.6	87	5.6	<3
Equatorial Pacific	0°02′S, 150°42′E and 1°35′N, 149°34′E	4	7 Feb. 1995	8AU	0.21	0.99	35	<0.2	8.3	92	2.3	<4
Transitional north Pacific	31°20′N, 140°25′E and 32°22′N, 140°13′E	4	12 Feb. 1995	8AU	0.23	0.52	3.2	<3	5.4	94	0.90	<3
Coastal												
Tokyo Bay	35°08′N, 139°46′E	1	30 July 1996	8AU	11.8	0.32	—	—	61	81	0.93	—
Otsuchi Bay	39°21′N, 141°59′E	2	2 June 1995	GF-75	7.2	0.26	23	2.7	21	67	3.0	1.1
	39°21′N, 141°59′E	5	5 June 1995	GF-75	4.1	0.26	37	2.3	20	72	1.8	1.0
	39°21′N, 141°59′E	10	17 May 1996	GF-75	4.2	4.7	—	—	33	37	86	—
	39°21′N, 141°59′E	10	20 May 1996	GF-75	3.6	2.6	—	—	29	23	15	—

[a] Comparisons of chlorophyll *a* (Chl. *a*) concentrations and cell abundances of cyanobacteria (Cyano) and bacteria are made between whole seawater (SW) and filtrates.
[b] Some samples were taken while the ship was steaming. In these cases, positions at the beginning and end of sampling are reported.
[c] 8AU, Micropore 8AU; GF-75, Advantec GF-75. Nominal pore sizes are given in Materials and Methods.
[d] Percentage of the chlorophyll *a* concentration or the number of cells (cyanobacteria or bacteria) in whole seawater that passed through the prefiltration filter.
[e] Ratio of carbon in phytoplankton to total organic carbon in the concentrated samples; calculations were based on a carbon-to-chlorophyll *a* ratio of 50 and a cyanobacterial carbon content of 200 fg cell^{-1}. Chlorophyll *a* concentrations and cyanobacterial abundances of concentrated oceanic samples were calculated from those in prefiltered samples by assuming that the concentration factors of chlorophyll *a* and cyanobacteria are the same as that of bacteria (see the text).
[f] —, not determined.

and nitrogen. The total number of bacteria finally recovered was 1 to 26% of the total number of bacteria in the original seawater (Table 2).

In order to examine the possible contribution of phytoplankton to total organic carbon in the concentrated samples, the amount of phytoplankton carbon was estimated from the chlorophyll *a* concentration and the abundance of cyanobacteria (Table 1). Here we assumed that the carbon-to-chlorophyll *a* weight ratio is 50 and the carbon content of cyanobacteria is 200 fg cell^{-1} (9), although the carbon content of phytoplankton may be variable depending on its physiological state. Since the chlorophyll *a* concentrations of most oceanic samples were close to the detection limit after prefiltration (Table 1), we did not determine chlorophyll *a* concentrations in the concentrated samples. By assuming the same recovery level for bacteria and phytoplankton during the concentration procedure, the contribution of phytoplankton carbon to total carbon in the concentrate was estimated to be minor (<11%) for both oceanic and coastal samples, except for the 1996 Otsuchi Bay samples (Table 1).

We examined if bacterium-sized detritus (submicron particles; size range, 0.4 to 1 μm) (27) contributed to total organic carbon and nitrogen in the concentrates. Because previous studies have shown that submicron particles are less dense than bacteria and that these particles can be separated by centrifugation (27), we compared bacterial carbon and nitrogen contents in the precipitates with those in the samples before centrifugation, using the Otsuchi Bay samples. We found that 63 to 80% of bacteria in the samples concentrated by tangential flow filtration were precipitated by centrifugation ($n = 3$). The carbon and nitrogen contents of the samples concentrated by tangential flow filtration were 96 to 109% of those of the centrifuged samples ($n = 3$), indicating that contamination with nonliving organic particles of low density was minor in the Otsuchi Bay samples.

Carbon and nitrogen contents of marine bacterial assemblages. Values for bacterial carbon and nitrogen contents are summarized in Table 3. These quotients were calculated by using the concentrations of organic carbon or nitrogen as determined by the HTCO method and the numbers of bacterial cells in the concentrated samples. The bacterial carbon contents of oceanic samples varied between 5.9 and 23.5 fg cell^{-1} (average ± SD, 12.4 ± 6.3 fg of C cell^{-1}; $n = 6$). The corresponding values in coastal samples ranged from 15.7 to 47.9 fg cell^{-1} (average ± SD, 30.2 ± 12.3 fg of C cell^{-1}; $n = 5$), significantly higher than those in oceanic samples ($P < 0.03$ by the two-tailed Student *t* test). Estimates of nitrogen content for oceanic samples were 1.2 to 3.9 fg cell^{-1} (average ± SD, 2.1 ± 1.1 fg of N cell^{-1}), significantly ($P < 0.002$) lower than those of coastal samples (range, 3.7 to 7.3 fg of N cell^{-1}; average ± SD, 5.8 ± 1.5 fg of N cell^{-1}). The atomic ratios of carbon to nitrogen content (C:N ratios) in oceanic samples varied between 5.4 and 8.3, with an average value of 6.8 (SD = 1.2; $n = 6$) (Table 3). The C:N ratios for coastal bacterial assemblages ranged from 5.0 to 7.7 (average ± SD, 5.9 ± 1.1; $n = 5$), and there was no significant ($P > 0.2$) difference between oceanic and coastal samples. The values for carbon and nitrogen contents and C:N ratios obtained at short intervals in the Otsuchi Bay were quite similar to each other (Table 3).

Accounting for error propagations, estimates bear analytical errors (SDs) in the range of 0.4 to 8.4 fg cell^{-1} (CV, 7 to 38%), 0.1 to 1.9 fg cell^{-1} (CV, 7 to 38%), and 0.1 to 1.0 (CV, 1 to

TABLE 2. Concentration of bacteria by tangential flow filtration[a]

Sample	Seawater		Bacteria			% of bacteria recovered in:			
	Initial vol (liters)	Concn factor	Initial no. (10^5 ml^{-1})	No. in retentate (10^7 ml^{-1})	Concn factor	Retentate[b]	Permeate[c]	Retentate + permeate[d]	Ultraconcentrated sample[e]
Oceanic									
Southern (south)	95	103	2.7	0.39	14	14	32	46	11
Southern (north)	300	268	5.7	13	225	84	12	96	16
Subtropical Pacific (south)	448	446	5.3	1.3	24	5.3	7.3	13	4
Subtropical Pacific (north)	550	546	5.8	1.8	31	5.6	68	74	1
Equatorial Pacific	634	649	7.6	9.6	126	19	51	70	9
Transitional north Pacific	320	307	5.0	0.69	14	4.5	ND[f]	ND	3
Coastal									
Tokyo Bay[g]	50	95	49	11	21	22	31	53	18
Otsuchi Bay									
2 June 1995	140	157	14	19	131	83	0.02	83	26
5 June 1995	190	110	14	12	88	43	0.03	43	23
17 May 1996	95	88	12	0.84	6.9	7.8	1.5	9.3	2
20 May 1996	100	98	7.0	0.89	13	14	1.8	16	2

[a] Bacterial cell abundances in prefiltered seawater (initial), the concentrated fraction (retentate), and the filtrate fraction (permeate) are compared.
[b] (Total number of cells in retentate/total number of cells in prefiltered seawater) × 100.
[c] (Total number of cells in permeate/total number of cells in prefiltered seawater) × 100.
[d] [(Total number of cells in retentate + total number of cells in permeate)/total number of cells in prefiltered seawater] × 100.
[e] (Total number of cells in the concentrate used for measuring carbon and nitrogen/total number of cells in raw seawater) × 100.
[f] ND, not determined.
[g] A hollow-fiber cartridge filtration system was used to concentrate bacteria.

10%) for carbon content, nitrogen content, and C:N ratio, respectively. There was no systematic difference ($P > 0.05$) in the precision of these estimates between coastal and oceanic samples.

DISCUSSION

The method described in this paper has advantages over previous methods of estimating bacterial biomass in seawater. First, our estimation of bacterial biomass does not rely on biovolume and the carbon-to-biovolume ratio of bacterial cells. The validity of the carbon-to-biovolume ratio of natural bacterial populations has not been fully established due to the difficulty of measuring bacterial cell size accurately (28, 34). Second, our method does not require preincubation of seawater samples, which may result in changes in bacterial physiology and the taxonomic compositions of the samples during incubation (15, 22, 34). The use of the large-scale tangential flow filtration technique (2), combined with sensitive detection of carbon and nitrogen by HTCO analysis (35), made it possible to determine directly, for the first time, the elemental compositions of natural populations of bacteria in oligotrophic oceans. In the following discussion, we first examine potential problems of our method and then discuss the implications of our results for the assessment of microbial biomass in diverse marine environments.

Methodological problems. Possible errors involved in our estimation of bacterial biomass include inclusion of nonbacterial organic matter such as phytoplankton and detritus in the concentrate. If such organic matter were included in the concentrate, our estimates of bacterial carbon and nitrogen content would be too high. However, in all the samples except for those taken from the Otsuchi Bay on 17 May 1996, the contribution of phytoplankton carbon (estimated from the chlorophyll *a* concentration and cyanobacterial abundance) was minor (<15% of total organic carbon in the concentrate [Table 1]). Although we did not count the abundance of prochlorophytes, which are dominant in the oligotrophic oceans (10), our estimation of phytoplankton carbon from chlorophyll *a* probably accounts for the contribution by prochlorophytes; prochlorophytes are generally retained by GF/F filters (23, 37). We also examined if bacterium-sized detritus (submicron particles) (27) contributed to our measurement of bacterial car-

TABLE 3. Bacterial carbon and nitrogen contents for concentrated samples

Sample	Carbon (fg cell^{-1})	Nitrogen (fg cell^{-1})	C:N ratio (atom atom^{-1})
Oceanic			
Southern, south (65°S)	23.5	3.9	7.0
Southern, north (48°S)	6.5	1.2	6.3
Subtropical Pacific, south (15°S)	12.5	1.8	8.1
Subtropical Pacific, north (15°N)	12.8	1.8	8.3
Equatorial Pacific	5.9	1.2	5.7
Transitional north Pacific	13.3	2.9	5.4
Avg[a]	12.4 ± 6.3	2.1 ± 1.1	6.8 ± 1.2
Coastal			
Tokyo Bay	47.9	7.3	7.7
Otsuchi Bay, 2 June 1995	15.7	3.7	5.0
Otsuchi Bay, 5 June 1995	22.1	5.0	5.2
Otsuchi Bay, 17 May 1996	33.1	6.5	5.9
Otsuchi Bay, 20 May 1996	32.0	6.6	5.7
Avg[b]	30.2 ± 12.3	5.8 ± 1.5	5.9 ± 1.1

[a] Mean ± SD for six oceanic samples.
[b] Mean ± SD for five coastal samples.

TABLE 4. Carbon contents and C:N ratios of natural marine bacteria reported in the literature

Habitat	Method(s)[a]		Carbon content[b] (fg cell^{-1})	C:N ratio (atom atom^{-1})	Source or reference
	Preincubation	Measurements			
Estuary and coastal					
Knebel Vig	w/o	X-ray	7–12	4.2–4.5	17
Raunefjorden	w/o	X-ray	9–19	3.8–4.2	17
Scripps pier	U	Calc.[c]	10.4–53.3	ND[d]	38
Crane Neck	U	CHN, EFM	15.4–24.8	2.9–5.0	29
Otsuchi Bay	U	CHN, PC	(17.3–53.3)	5.00–14.2	26
Roskilde Fjord	U	CHN, EFM	(30–43)	ND	4
Roskilde Fjord	E	CHN, EFM	(26–84)	ND	4
Aburatsubo Bay	U	CHN, PC	(31.4–99.8)	3.99–5.41	26
Santa Rosa Sound	U	CHN, EFM	(54–90)	2.83–6.07	28
Puddefjorden	E	CHN, EFM	106–214	ND	7
Gulf of Mexico	U	CHN, EFM	(150)	10.29	28
Perdido Bay	U	CHN, EFM	(200–260)	8.77–9.92	28
Otsuchi Bay	w/o	HTCO, EFM	15.7–33.1	5.0–5.9	This study
Tokyo Bay	w/o	HTCO, EFM	47.9	7.7	This study
Ocean					
Subtropical Pacific	w/o	Calc.[e]	2.45–8.76	ND	12
Southern	U	CHN, EFM	(44–52)	ND	6
Equatorial Pacific	w/o	HTCO, EFM	5.9	5.7	This study
Southern (north)	w/o	HTCO, EFM	6.5	6.3	This study
Subtropical Pacific	w/o	HTCO, EFM	12.5–12.8	8.1–8.3	This study
Transitional north Pacific	w/o	HTCO, EFM	13.3	5.4	This study
Southern (south)	w/o	HTCO, EFM	23.5	7.0	This study

[a] w/o, natural water; U, in situ water culture; E, enriched culture; X-ray, X-ray microanalysis; EFM, epifluorescence microscopy; PC, particle counting.
[b] Values in parentheses were calculated from carbon-to-biovolume ratios.
[c] Calculation from macromolecules.
[d] ND, not determined.
[e] Calculation from ATP.

bon. Results from the centrifugation experiment showed that bacteria and organic carbon were precipitated at the same ratio (the difference was within 5%), indicating that the contribution of submicron particles to the measurement of total organic carbon in the concentrates was minor.

After concentration by tangential flow filtration, recovered bacteria were 5 to 84% (average, 27%) of total bacteria in prefiltered seawater (Table 2). This result is consistent with previous observations that the recovery of bacteria and picoplankton from a large volume of seawater (50 to 8,000 liters) by tangential flow filtration is typically 37 to 80% (2, 13, 19, 21). Low levels of recovery would be a consequence of (i) passage of a portion of bacterial populations through the filters or (ii) incomplete removal of bacterial populations which firmly attached to filters, or both. The first factor (passage of bacteria through filters) was significant only for some oceanic samples from the equatorial and subtropical Pacific; 51 to 68% of bacteria were found in the permeate. We hypothesize that relatively small bacteria were abundant in these oligotrophic waters (32) and that these small bacteria passed through the filters. If this is correct, the carbon and nitrogen contents of bacteria that we determined for these samples would be too large. Concerning the second factor (incomplete removal), we know little about the mechanism of recovery of bacterial populations from tangential flow filtration systems. In our experience, bacteria recovered in the retentate tend to be in the form of large aggregates, which are probably formed during vigorous flushing (see Materials and Methods). We assume that selective accumulation of specific bacterial populations in these large aggregates did not occur, i.e., that bacterial recovery was a nonselective process. Consistent with this notion, Giovannoni et al. (21) observed that the taxonomic composition of picoplankton in the concentrate made by tangential flow filtration did not differ from that in original seawater.

Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. Our estimates of bacterial carbon content in samples collected from distant and diverse marine ecosystems (Table 1) varied widely (5.9 to 47.9 fg of C cell^{-1} [Table 3]). This result suggests that the geographical and seasonal variability of bacterial carbon content is quite large. Notably, we found that the carbon content of oceanic samples (average $\pm$ SD, 12.4 $\pm$ 6.3 fg cell^{-1}) is much lower than that of coastal samples (30.2 $\pm$ 12.3 fg cell^{-1}), even though the carbon content of oceanic bacteria could be overestimated, as mentioned above. This pattern probably can be explained by differences in the physiological state of bacterial populations between oligotrophic and productive waters. In support of this hypothesis, previous work has shown that bacteria growing faster under rich nutritional conditions are generally larger than those under conditions of starvation (34).

Table 4 summarizes the carbon contents of marine bacteria reported in the literature. Our estimates in coastal environments (15.7 to 47.9 fg of C cell^{-1}) are within the range previously reported at a Long Island beach (20 fg of C cell^{-1}) (29) and the Otsuchi Bay (17.3 to 53.3 fg of C cell^{-1}) (26). Lower values (7 to 19 fg of C cell^{-1}) have been obtained by X-ray microanalysis for samples collected in Raunefjorden and Knebel Vig (17). Christian and Karl (12) estimated indirectly, by a least-squares inverse method, that bacterial carbon content was close to 10 fg of C cell^{-1} in the subtropical Pacific Ocean. Our estimate of 13 fg of C cell^{-1} in the subtropical Pacific Ocean is consistent with their estimates. Caron et al. (9) suggested a bacterial carbon content of 15 fg of C cell^{-1} in the

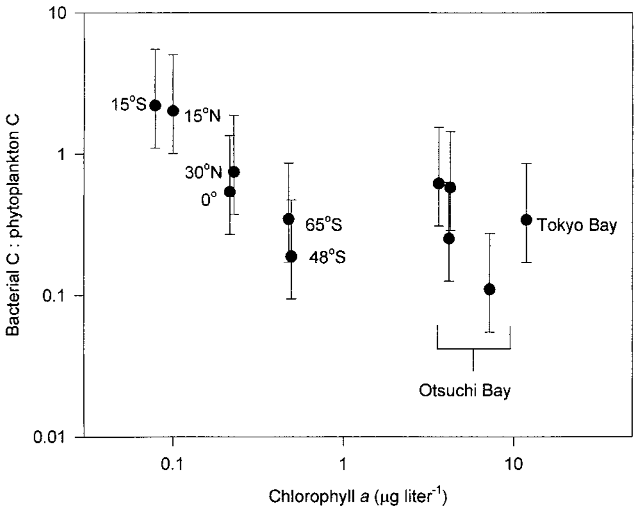

FIG. 1. Ratio of bacterial carbon biomass to phytoplankton biomass in the study area. Bacterial carbon was estimated by using the data in Table 3. Phytoplankton carbon was estimated by assuming that the carbon-to-chlorophyll *a* ratio is 50. Error bars indicate the ranges of estimates with carbon-to-chlorophyll *a* ratios ranging from 20 to 100. Regions for which results are shown are indicated either by latitudinal positions (for oceanic samples) or by geographic names (see Table 1). Note that there are four results from the Otsuchi Bay.

Sargasso Sea, and our average estimate of 12.4 fg of C cell^{-1} for oceanic environments is also consistent with their value.

We found that C:N ratios of bacteria varied in the range of 5.0 to 8.3 (average ± SD, 6.4 ± 1.2; CV, 19%; $n = 11$) (Table 3). This result is consistent with those of previous studies, which reported natural bacterial C:N ratios in aquatic environments that averaged around 5 to 7 (Table 4). Although the carbon and nitrogen contents of bacteria in coastal samples were significantly different from those in oceanic samples, no difference in C:N ratio was detected. Some researchers concluded that the bacterial C:N ratio was unaffected (CV, 15 to 36%) by the substrate C:N ratio (7, 24, 34). Our data support the hypothesis that the bacterial C:N ratio is relatively invariant in marine environments.

Ratio of carbon in bacteria to carbon in phytoplankton in marine environments. We estimated bacterial carbon biomass in the surface waters of several marine environments by using the obtained carbon content and abundance and then compared it with phytoplankton biomass (Fig. 1). Phytoplankton carbon was estimated from the chlorophyll *a* concentration by assuming carbon-to-chlorophyll *a* weight ratios of 20 to 100. Estimates derived by using a fixed carbon-to-chlorophyll *a* weight ratio of 50 will be used in the following discussion to facilitate comparison with other studies (11, 16, 25). Note that, for estimating bacterial biomass, we use the carbon content (5.9 to 47.9 fg of C cell^{-1}) which was directly determined for natural populations in each region, whereas previous studies set the value at 20 fg of C cell^{-1}. Our estimates of bacterial biomass were 3.9 to 8.5 μg of C liter^{-1} in oceanic waters and 33 to 290 μg of C liter^{-1} in coastal waters. The ratios of bacterial carbon biomass to phytoplankton biomass were 1.7 and 1.9 for two samples in the subtropical Pacific Ocean, the only region where bacterial carbon exceeded phytoplankton carbon. In other regions of the oceans, the carbon biomass ratios range from 0.2 to 0.6, which is similar to those for coastal regions (0.1 to 0.5). Cho and Azam (11) noted that in the North Pacific Gyre, the ratio of bacterial carbon biomass to phytoplankton biomass increased with a decrease in chlorophyll *a* concentration when the chlorophyll *a* concentration was <0.2 μg liter^{-1}, and the carbon biomass ratio was as high as 8. (See also Simon and Azam [38].) Ducklow and Carlson (16) also reported that bacterial carbon exceeded phytoplankton carbon when the chlorophyll *a* concentration was below ca. 0.1 to 1 μg liter^{-1}. Although our estimates support the previous propositions, i.e., that bacterial carbon biomass contributes significantly to total biomass in the oceans (8, 11, 16, 20, 39), the degree of the predominance is not as large as previously thought. Buck et al. (8) suggested that the dominance of bacterial biomass should not be recognized as a general rule in oceanic waters, and our results are consistent with their opinion. On the other hand, our results suggest that the use of a fixed factor (20 fg of C cell^{-1}) could result in great underestimation of bacterial biomass in productive coastal environments. Previous works (16, 39) showed a negative correlation between the logarithm of the chlorophyll *a* concentration and the logarithm of the ratio of bacterial carbon biomass to phytoplankton biomass. Our data also yield a strong negative correlation between these variables if we assume that a fixed factor of bacterial carbon content is applicable to all the investigated regions ($r = -0.90$; $P < 0.0002$; $n = 11$). However, if we use our measured values for bacterial carbon content for each region, the correlation is not significant ($P > 0.05$). Therefore, the relationships between the ratios of bacterial carbon biomass to phytoplankton biomass and productivity in marine systems should be reevaluated by taking into account the regional variability of bacterial carbon content.

ACKNOWLEDGMENTS

We thank the captain and crew of the R.V. *Hakuho-maru* and the scientists on board for help with CTD sampling. We also thank E. Tanoue for lending us the Pellicon system and Organo Corp. for providing Micropore 8AU. We are also grateful to H. Otobe and K. Morita for help in collecting samples in the Otsuchi Bay and to K. Ishikawa and S. Sukisaki for help with sampling in the Tokyo Bay.

This work was supported by a grant from the Ministry of Education, Science, Sports, and Culture of Japan (07404044).

REFERENCES

1. **Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad.** 1983. The ecological role of water-column microbes in the sea. Mar. Ecol. Prog. Ser. **10:**257–263.
2. **Benner, R.** 1991. Ultra-filtration for the concentration of bacteria, viruses, and dissolved organic matter. Geophys. Monogr. **63:**275–280.
3. **Billen, G., C. Joiris, L. Meyer-Reil, and H. Lindeboom.** 1990. Role of bacteria in the North Sea ecosystem. Neth. J. Sea Res. **4:**265–293.
4. **Bjørnsen, P. K.** 1986. Automatic determination of bacterioplankton biomass by image analysis. Appl. Environ. Microbiol. **51:**1199–1204.
5. **Bjørnsen, P. K., and B. Riemann.** 1988. Towards a quantitative stage in the study of microbial processes in pelagic carbon flows. Ergeb. Limnol. **31:**185–193.
6. **Bjørnsen, P. K., and J. Kuparinen.** 1991. Determination of bacterioplankton biomass, net production and growth efficiency in the Southern Ocean. Mar. Ecol. Prog. Ser. **71:**185–194.
7. **Bratbak, G.** 1985. Bacterial biovolume and biomass estimations. Appl. Environ. Microbiol. **49:**1488–1493.
8. **Buck, K. R., F. P. Chavez, and L. Campbell.** 1996. Basin-wide distributions of living carbon components and the inverted trophic pyramid of the central gyre of the North Atlantic Ocean, summer 1993. Aquat. Microb. Ecol. **10:**283–298.
9. **Caron, D. A., H. G. Dam, P. Kremer, E. J. Lessard, L. P. Madin, T. C. Malone, J. M. Napp, E. R. Peele, M. R. Roman, and M. J. Youngbluth.** 1995. The contribution of microorganisms to particulate carbon and nitrogen in surface waters of the Sargasso Sea near Bermuda. Deep-Sea Res. **42:**943–972.
10. **Chisholm, S. W., R. J. Olson, E. R. Zettler, R. Goericke, J. B. Waterbury, and N. A. Welschmeyer.** 1988. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature **334:**340–343.
11. **Cho, B. C., and F. Azam.** 1990. Biogeochemical significance of bacterial biomass in the ocean's euphotic zone. Mar. Ecol. Prog. Ser. **63:**253–259.
12. **Christian, J. R., and D. M. Karl.** 1994. Microbial community structure at the U.S.-Joint Global Ocean Flux Study Station ALOHA: inverse methods for

estimating biochemical indicator ratios. J. Geophys. Res. **99:**14269–14276.
13. **Coffin, R. B., D. J. Velinsky, R. Devereux, W. A. Price, and L. Cifuentes.** 1990. Stable carbon isotope analysis of nucleic acids to trace sources of dissolved substrates used by estuarine bacteria. Appl. Environ. Microbiol. **56:**2012–2020.
14. **Cole, J. J., S. Findlay, and M. L. Pace.** 1988. Bacterial production in fresh and saltwater ecosystems: a cross-system overview. Mar. Ecol. Prog. Ser. **43:**1–10.
15. **DeLong, E. F.** 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. USA **89:**5685–5689.
16. **Ducklow, H. W., and C. A. Carlson.** 1992. Oceanic bacterial production, p. 113–181. *In* K. C. Marshall (ed.), Advances in microbial ecology. Plenum Press, New York, N.Y.
17. **Fagerbakke, K. M., M. Heldal, and S. Norland.** 1996. Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria. Aquat. Microb. Ecol. **10:**15–27.
18. **Fry, J. C.** 1988. Determination of biomass, p. 27–72. *In* B. Austin (ed.), Methods in aquatic bacteriology. John Wiley & Sons, Inc., New York, N.Y.
19. **Fuhrman, J. A., D. E. Comeau, Å. Hagström, and A. M. Chan.** 1988. Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Appl. Environ. Microbiol. **54:**1426–1429.
20. **Fuhrman, J. A., T. D. Sleeter, C. A. Carlson, and L. M. Proctor.** 1989. Dominance of bacterial biomass in the Sargasso Sea and its ecological implications. Mar. Ecol. Prog. Ser. **57:**207–217.
21. **Giovannoni, S. J., E. F. DeLong, T. M. Schmidt, and N. R. Pace.** 1990. Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. Appl. Environ. Microbiol. **56:**2572–2575.
22. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature **345:**60–63.
23. **Goericke, R., and N. A. Welschmeyer.** 1993. The marine prochlorophyte *Prochlorococcus* contributes significantly to phytoplankton biomass and primary production in the Sargasso Sea. Deep-Sea Res. **40:**2283–2294.
24. **Goldman, J. C., D. A. Caron, and M. R. Dennett.** 1987. Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. Limnol. Oceanogr. **32:**1239–1252.
25. **Kirchman, D. L., R. G. Keil, M. Simon, and N. A. Welschmeyer.** 1993. Biomass and production of heterotrophic bacterioplankton in the oceanic subarctic Pacific. Deep-Sea Res. **40:**967–988.
26. **Kogure, K., and I. Koike.** 1987. Particle counter determination of bacterial biomass in seawater. Appl. Environ. Microbiol. **53:**274–277.
27. **Koike, I., S. Hara, K. Terauchi, and K. Kogure.** 1990. Role of sub-micrometre particles in the ocean. Nature **345:**242–244.
28. **Kroer, N.** 1994. Relationships between biovolume and carbon and nitrogen content of bacterioplankton. FEMS Microbiol. Ecol. **13:**217–224.
29. **Lee, S., and J. A. Fuhrman.** 1987. Relationships between biovolume and biomass of naturally derived marine bacterioplankton. Appl. Environ. Microbiol. **53:**1298–1303.
30. **Montesinos, E., I. Esteve, and R. Guerrero.** 1983. Comparison between direct methods for determination of microbial cell volume: electron microscopy and electronic particle sizing. Appl. Environ. Microbiol. **45:**1651–1658.
31. **Moriarty, D. J. W., M. Bianchi, and V. Talbot.** 1997. Bacterial productivity and organic matter flux in the Southern Ocean and in the Antarctic intermediate water and mode water of the Indian Ocean. Deep-Sea Res. Part II **44:**1005–1015.
32. **Morita, R. Y.** 1997. Bacteria in oligotrophic environments—starvation-survival lifestyle, p. 195–197. International Thomson Publishing, New York, N.Y.
33. **Nagata, T.** 1986. Carbon and nitrogen content of natural planktonic bacteria. Appl. Environ. Microbiol. **52:**28–32.
34. **Nagata, T., and Y. Watanabe.** 1990. Carbon- and nitrogen-to-volume ratios of bacterioplankton grown under different nutritional conditions. Appl. Environ. Microbiol. **56:**1303–1309.
35. **Ogawa, H., R. Fukuda, and I. Koike.** Vertical distributions of dissolved organic carbon and nitrogen in the Southern Ocean. Submitted for publication.
36. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943–948.
37. **Sieracki, M. E., E. M. Haugen, and T. L. Cucci.** 1995. Overestimation of heterotrophic bacteria in the Sargasso Sea: direct evidence by flow and imaging cytometry. Deep-Sea Res. **42:**1399–1409.
38. **Simon, M., and F. Azam.** 1989. Protein content and protein synthesis rates of planktonic bacteria. Mar. Ecol. Prog. Ser. **51:**201–213.
39. **Simon, M., B. C. Cho, and F. Azam.** 1992. Significance of bacterial biomass in lakes and the ocean: comparison to phytoplankton biomass and biogeochemical implications. Mar. Ecol. Prog. Ser. **86:**103–110.
40. **Suzuki, R., and T. Ishimaru.** 1990. An improved method for the determination of phytoplankton chlorophyll using *N*,*N*-dimethylformamide. J. Oceanogr. Soc. Jpn. **46:**190–194.
41. **Viles, C. L., and M. E. Sieracki.** 1992. Measurement of marine picoplankton cell size by using a cooled, charge-coupled device camera with image-analyzed fluorescence microscopy. Appl. Environ. Microbiol. **58:**584–592.
42. **Wangersky, P. J.** 1993. Dissolved organic carbon methods: a critical review. Mar. Chem. **41:**61–74.
43. **Watson, S. W., T. J. Novitsky, H. L. Quinby, and F. W. Valois.** 1977. Determination of bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. **33:**940–946.
44. **Yamasaki, A., H. Fukuda, R. Fukuda, T. Miyajima, T. Nagata, H. Ogawa, and I. Koike.** 1998. Submicrometer particles in northwest Pacific coastal environments: abundance, size distribution, and biological origins. Limnol. Oceanogr. **43:**536–542.